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(54) Title: DETECTION OF MINIMAL NUMBERS OF NEOPLASTIC CELLS CARRYING DNA TRANSLOCATIONS BY DNA SEQUENCE AMPLIFICATION		
(57) Abstract <p>The method of the present invention typically includes the following steps. (1) Incubating a mixture comprising: (a) deoxyribonucleotide triphosphates including deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytosine triphosphate and deoxyguanosine triphosphate; (b) DNA from cells, most particularly lymphoid cells from blood, bone marrow or lymph node, of the individual; (c) DNA polymerase, preferably the Klenow fragment of E. coli DNA polymerase or Thermus aquaticus DNA polymerase; (d) a first oligonucleotide primer, said first primer being identical to an upstream nucleotide sequence flanking coding DNA translocation derived from one chromosome; and (e) a second oligonucleotide primer, said second primer being complementary to a downstream nucleotide sequence flanking the coding DNA translocation derived from the other chromosome. The incubating is under conditions allowing annealing of the primers to the crossover site of translocation and synthesis of coding or non-coding translocation DNA sequences and a substantial portion of flanking DNA sequences. The incubation is then terminated by DNA denaturation. (2) The cycles of annealing, synthesis and denaturation are then repeated numerous times (preferably 20-50) to facilitate duplications of the original and previously synthesized translocated and substantial flanking DNA sequences. The repetition is carried out in a manner allowing exponential amplification of DNA sequences. (3) The exponentially amplified DNA is probed for the presence of oligonucleotide sequences characterizing the translocation region. Positive probings indicate the presence of neoplastic cells in the sample of the individual's cells, since DNA characterizing said neoplastic cells was specifically amplified and probed.</p>		

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DETECTION OF MINIMAL NUMBERS OF NEOPLASTIC
CELLS CARRYING DNA TRANSLOCATIONS
10 BY DNA SEQUENCE AMPLIFICATION

High frequency of recurrence is one of the major
15 problems in cancer treatment. Relapse from clinically
undetectable residual disease is the most likely
mechanism. Detection of minimal disease is extremely
difficult since tumor specific markers are not readily
available. Molecular technology has provided a means to
20 demonstrate residual disease by identifying clonal
rearrangement patterns that are present in malignant
hematopoietic cells (1). Southern blot hybridization
detects neoplastic cells at levels as low as 1% of the
total number of cells (2). However, one of the major
25 drawbacks in this situation is that it is difficult to be
certain that faint nongermline bands indeed represent
clonal rearrangements. Furthermore, no rearranged bands
can be detected in cases in which the concentration of
neoplastic cells is below 1%. Theoretically this occurs
30 frequently while patients are in remission.

The karyotypic abnormality, t(14;18)(q32;q21), has
been observed in approximately 90% of human follicular
lymphomas (3-5). This translocation frequently results in
35 rearrangement of a putative oncogene "bcl-2" which resides
at chromosome 18 band q21 (6). In the majority of cases

with the t(14;18), the molecular breakpoints on chromosome 18q21 cluster within a 4.3 kilobase (kb) Hind III restriction fragment or more specifically a 2.8 kb EcoRI-Hind III restriction fragment which has recently been
5 designated the t(14;18) major breakpoint cluster region (mbr) (7-11). DNA sequencing of the crossover sites revealed that breakpoints on chromosome 18q21 were clustered within 150 base pairs (bp) of each other and breakpoints on chromosome 14 were located close to the 5'
10 end of one of the joining (J) segments (J1 to J6) of the immunoglobulin heavy chain locus (JH) (11-13).

Saiki et al. have recently utilized a new technique, sequence amplification by polymerase chain reaction (PCR),
15 to diagnose sickle cell anemia prenatally (14). This technique is mainly applicable to genetic disorders with a point DNA mutation. It is difficult to apply PCR to the detection of chromosomal translocation because of the variability of molecular breakpoints on chromosomes. The
20 present invention describes a method of utilizing the PCR technique to amplify DNA sequences flanking the crossover site of a chromosomal translocation such as t(14;18) which is characteristic of a tumor. Since cells carrying the subject t(4;18) translocation are unique to malignant
25 hematopoietic cells, detection and amplification of such sequences can be used as evidence for minimal residual disease. The present invention also demonstrates the unique consequence of applying the PCR technique to a chromosomal translocation, i.e., preferential
30 amplification of the hybrid DNA sequences of the chromosomal translocation, but not the normal DNA sequence.

The present invention involves a method for detecting
35 minimal numbers of neoplastic cells in an individual. These neoplastic cells are typically residual cells from a

lymphoma incompletely eradicated by antitumor therapy.
The subject neoplastic cells of the present invention may
be characterized by a DNA translocation related to a
specific chromosomal breakpoint clustering region. A
5 neoplasm to which the present invention may be applied is
most typically a human follicular lymphoma although it may
also be characterized as a non-Hodgkin's lymphoma, a pre-
B-cell or B-cell leukemia, a B-cell lymphoma, a large cell
lymphoma, a diffuse large cell lymphoma or a small
10 noncleaved cell lymphoma.

The method of the present invention typically
includes the following steps.

- 15 (1) Incubating a mixture comprising:
- (a) deoxyribonucleotide triphosphates including
deoxyadenosine triphosphate, deoxythymidine
triphosphate, deoxycytosine triphosphate
20 and deoxyguanosine triphosphate;
 - (b) DNA from cells, most particularly lymphoid
cells from blood, bone marrow or lymph node
of the individual;
 - 25 (c) DNA polymerase, preferably the Klenow
fragment of E. coli DNA polymerase or
Thermus aquaticus DNA polymerase.
 - 30 (d) a first oligonucleotide primer, said first
primer being identical to an upstream
nucleotide sequence flanking the coding DNA
translocation, derived from one chromosome
such as chromosome 18q21..

35

(e) a second oligonucleotide primer, said second primer being complementary to a downstream nucleotide sequence flanking the coding DNA translocation derived from the other chromosome, such as chromosome 14q32. (The primers are present in the incubation mixture in excess of that needed to bind complementary oligonucleotide sequences of the DNA and are preferably at about a 1 micromolar (μM) concentration.).

The incubating is under conditions allowing annealing of the primers to the crossover site of the chromosomal translocation DNA sequences and a substantial portion of flanking DNA sequences.

(2) The incubating is then terminated by DNA denaturation. The cycles of annealing, synthesis and denaturation are repeated numerous times (preferably 20-50) to facilitate duplications of the original and previously synthesized translocated and substantial flanking DNA sequences. The repetition is carried out in a manner allowing exponential amplification of DNA sequences extending from primers and including a DNA region complementary to the other oligonucleotide primers. This amplification is an extension of each primer to and including a post-translocational DNA sequence complementary to the other member of the primer set being used.

(3) The exponentially amplified DNA is probed for the presence of oligonucleotide sequences characterizing the translocation region. Elongation of each primer in this system

eventually involves the addition thereto of a coding or non-coding translocation DNA sequence usually terminating in the oligonucleotide complementary to the other member of the primer set being utilized. Positive probings indicate the presence of neoplastic cells in the sample of the individual's cells, since DNA characterizing said neoplastic cells was specifically amplified and probed.

10

The above-described procedure may likewise be directed to detection of neoplastic cells characterized by DNA translocation related to more than one specific breakpoint clustering regions. The method described above could be modified as follows where more than one breakpoint clustering region may be being amplified and probed. The incubated mixture could have at least two sets of oligonucleotide primers, each set flanking a particular breakpoint clustering region. Each set of oligonucleotide primers consists of a first oligonucleotide primer and a second oligonucleotide primer, said first oligonucleotide primer being identical to an upstream coding nucleotide sequence flanking a particular DNA breakpoint clustering region of a chromosomal translocation and said second primer being complementary to a downstream coding nucleotide sequence flanking the same particular DNA breakpoint clustering region of this translocation. The mixture of primers, DNA from cells, deoxynucleotide triphosphates and DNA polymerase and the incubating is again under conditions facilitating annealing of the primers to the crossover sites of the translocations and synthesis of the sequences of coding and non-coding translocation DNA and including a substantial portion of flanking DNA, such a substantial portion including a DNA portion complementary to the other member of the particular primer set being used. Again the

incubating is terminated by DNA denaturations. The annealing, synthesis and denaturation are repeated in a cyclic manner to facilitate further synthesis of the original DNA and of newly synthesized DNA, said repeating
5 being carried out in a manner allowing exponential amplification of DNA sequences between initiating oligonucleotide primers and sequences complementary to the other member of the primer set. Lastly, the exponentially amplified DNA is probed for the present of oligonucleotide
10 sequences characterizing the translocation of DNA sequence. The presence of neoplastic cells in the sample of the individual's cells are identified by positive probings. Such probings are most typically carried out by Southern blot or Dot blot procedures whose methodologies
15 are well-known to those skilled in the relevant arts. The method of the present invention involves in vitro synthesis of the DNA sequences at the crossover site of a tumor characteristic chromosomal translocation by primer extension and a substantial flanking DNA region including
20 that nucleotide sequence complementary to the primers.

The oligonucleotide primers utilized in the methods of the present invention are preferably complementary or identical to DNA sequences located within about 1000 bases
25 of the nearest chromosomal breakpoint clustering region. These oligonucleotide primers are further preferably between about 12 and about 20 nucleotides in length.

The method of the present invention most particularly
30 involves detection of neoplasia such as those having the t(14;18) translocation. These neoplasia characteristically have a major breakpoint clustering region in the majority of cases and a minor breakpoint clustering region in the minority of the cases, the major
35 breakpoint clustering region being the mbr region and minor breakpoint clustering region being the mcr region.

In cases where two breakpoint regions are to be analyzed, two sets of oligonucleotide primers are added to the above described incubation mixture and the probings are derived from the crossover sites of the translocated DNA.

5

The probing step of the present invention, as mentioned above, typically involves Southern blot analysis or Dot blot analysis. Such Southern blot analysis characteristically includes gel electrophoresis of the PCR
10 amplified DNA segments, transfer of electrophoresed fragments to a nitrocellulose filter or nylon filter and probing the filter of nucleotide sequences binding the probes of interest. Dot blot analysis is similar to the above procedure except that the PCR-amplified DNA is
15 denatured in situ and then blotted to the filter without gel electrophoresis

The method of the present invention preferably involves the detection of minimal numbers of neoplastic
20 cells with a chromosomal translocation. Such neoplastic cells, particularly those characterized by a t(14;18) chromosomal translocation are, most frequently human follicular lymphomas. Preferred subject neoplasia of the present invention may also be characterized as being a
25 non-Hodgkin's lymphoma, B-cell or pre-B cell leukemia, B-cell lymphoma, large cell lymphoma, diffuse large cell lymphoma or small noncleaved cell lymphoma.

Figure 1 schematically illustrates preferential PCR
30 amplification of the hybrid 18q21-JH DNA sequence.

Figure 2(A) shows a partial restriction enzyme map surrounding the mbr region of chromosome 18 band q21;
Figure 2(B) shows Southern blot hybridization with labeled
35 mbr probe of restriction enzyme digested DNA samples.

Figure 3 shows a Southern blot analysis of PCR-amplified genomic DNA with radioactively labeled primer 18q21(+) and primer J_H(-).

5 Figure 4 shows a Southern blot analysis of PCR-amplified genomic DNA with radioactively labeled oligonucleotide 18q21(+)II.

10 The present invention involves using the polymerase chain reaction (PCR) technique to amplify DNA sequences that flank the crossover site of a chromosomal translocation characteristic for follicular lymphomas, t(14;18)(q32;q21). This technique permitted the detection of cells carrying the t(14;18) hybrid DNA sequences at a
15 dilution of 1:100,000. The remission marrow and blood samples of patients with follicular lymphoma and the t(14;18) fail to show any abnormality by morphological examination and conventional Southern blot analysis. However, the t(14;18) hybrid DNA sequences were detected
20 by the PCR technique. Thus, the present invention described a highly sensitive tool to detect minimal residual cells carrying the t(14;18) and has the potential to identify a subpopulation of patients with subclinical disease. This invention can also be used for the early
25 detection and rapid diagnosis of a neoplasm with the t(14;18) as well as for the diagnosis of the t(14;18) by using a small amount of DNA sample (at the level of several nanograms (ng)).

30 Polymerase chain reaction (PCR) is a technique which allows exponential amplification of the target DNA sequences. It has been used for rapid diagnosis of certain hereditary genetic disorders in which a point DNA
35 mutation is present and each individual cell contains the same abnormality. The present invention involves the first application of PCR to a chromosomal translocation,

thus, resulting in preferential amplification of tumor specific hybrid DNA sequences in minimal number of cells carrying a chromosomal translocation among several hundred thousand normal cells.

5

Two synthetic oligonucleotides were prepared as primers for PCR usage. These two primers were expected to flank the crossover site of the t(14;18) in the majority of cases. Primer 18q21(+), 5'-TTTGACCTTTAG-3', was
10 identical to the sequences of the (+)-strand of chromosome 18q21 (11-13) and primer J_H(-), 3'-CAGAGGAGTCCA-5', was complementary to the sequences present in the 3' end of each (+)-strand J segment (15). Therefore, this primer
15 the breakpoint varies from J1 to J6 from case to case. In case of the t(14;18), copies of the (+)-strand hybrid 18q21-J_H DNA sequences were synthesized from primer 18q21(+) and copies of the (-)-strand hybrid 18q21-J_H DNA sequences were synthesized from primer J_H(-). New copies
20 of the (+)-strand and the (-)-strand hybrid 18q21-J_H DNA sequences in turn became templates of primer J_H(-) and primer 18q21(+) respectively. Therefore, DNA sequences flanking the crossover site of the t(14;18) were amplified exponentially as PCR was carried out (Figure 1A). In
25 contrast, such amplification could not be generated in cases without the t(14;18) because no new templates for the primers could be synthesized (Figure 1B). These two primers could also be used as probes to detect newly synthesized hybrid 18q21-J_H DNA sequences, which were
30 generated in case of the t(14;18), but not the new copies of 18q21(+) or J_H(-) DNA sequences which were synthesized in cases without the t(14;18). Another oligonucleotide 18q21(+)II, 5'-CACAGACCCACCCAGAGCCC-3', deduced from the mb region [27 bases 3' to the primer 18q21(+)], was used
35 as an "internal" probe to further confirm that the PCR amplified DNA segments contained the hybrid 18q21-J_H

sequences (11-13). Since 18q21(+)II derived from sequences of the (+)-strand chromosome 18q21, it cannot hybridize with sequences extended from primer 18q21(+). In contrast, it will hybridize with sequences extended
5 from primer J_H(-) in case of the t(14;18). Therefore, signals detected by the radiolabeled 18q21(+)II probe strongly indicate presence of the hybrid 18q21-J_H sequences.

10 Figure 1 schematically illustrates the mechanism by which PCR preferentially amplifies the hybrid 18q21-JH DNA sequences, but not the normal DNA sequences. As shown in Figure 1(A), in cases of the t(14;18), the hybrid 18q21-JH(+) and 18q21-JH(-) DNA sequences were synthesized from
15 primer 18q21(+) and primer JH(-) respectively. The primers are also complementary to the newly synthesized hybrid 18q21-JH(±) DNA sequences which, in turn, become templates for the primers. Therefore, exponential amplification of the hybrid 18q21-JH(±) DNA sequences are
20 generated, i.e., $Y = (1+E)^N$ where Y is the extent of yield, E is the mean efficiency per PCR cycle and N is the number of PCR cycles carried out. Presuming E=100% and N=20, the final yield is 2^{20} copies of hybrid 18q21-JH(±) DNA sequences.

25 As shown in Figure 1(B), in case of normal karyotype, the newly synthesized 18q21(+) and JH(-) DNA sequences can not be templates for the primers. Therefore, the final yield was calculated as the following formula: $y=2^n \times e$,
30 where y is the extent of yield, n is the number of PCR cycles and e is the mean efficiency per cycle.

By means of DNA sequencing, about 60% of follicular lymphomas have been shown to have breakpoints on
35 chromosome 18q21 tightly clustered within 150 base pairs of each other, which is named the molecular breakpoint hot

spot (11-13). Our primer 18q21(+) was derived from the (+)-strand DNA sequence of chromosome 18q21 immediately 5' to the molecular breakpoint hot spot. One "internal" probe, 18q21(+)II, was derived from the (+)-strand 18q21 DNA sequence 27 bases 3' to primer 18q21(+). The breakpoint on chromosome 14q32 is also very consistent from case to case. It always occurs at the 5' end of one of the J-segments (J1-J6) of the immunoglobulin heavy chain gene. Since the sequences at the 3' end of each J-segment are the same (15), primer J_H(-) with sequence derived from this region was made. Therefore, Primer J_H(-) would always anneal to the crossover site of the t(14;18) even though the breakpoints on chromosome 14q32 vary from J1 to J6 from case to case.

15

In order to establish the use of PCR to amplify the DNA sequences flanking the crossover sites of the t(14;18), three representative DNA samples were selected. Sample A was from a lymph node of follicular lymphoma with the t(14;18) breakpoint occurring within the mbr region. Sample B was from a lymph node of reactive lymphadenitis with normal karyotype. Sample C as from a lymph node of follicular lymphoma with the t(14;18) breakpoint 3' to the mbr region. These samples were analyzed by Southern blot hybridization with a human genomic DNA fragment specific for the mbr region of chromosome 18q21 (Figure 2A) (7,11). As shown in Figure 2B, Lanes 1 and 4, two rearranged bands were detected in Sample A, indicating the breakpoint on chromosome 18q21 occurring within mbr. In Sample C only one rearranged band was detected by restriction endonuclease SstI and no rearrangement was detected with HindIII (Figure 2B, Lanes 3 and 6). Therefore, the breakpoint occurred 3' to mbr (within the Hind III-SstI restriction fragment).

35

Figure 2(A) shows a partial restriction enzyme map surrounding the mbr region of chromosome 18 band q21. The solid bar represents the germline DNA structure of chromosome 18 band q21 (7). The horizontal line labeled as mbr indicates the t(14;18) major breakpoint cluster region and the probe used for Southern blot hybridization to map the chromosomal breakpoints of the t(14;18) on chromosome 18q21. The asterisk (*) indicates the t(14;18) breakpoint hot spot where primer 18q21(+) was deduced (1,13). (B) Southern blot hybridization with mbr probe. DNA's were digested with enzyme SstI or enzyme HindIII and size fractionated on a 0.8% agarose gel, transferred to a nylon filter, and hybridized with a radiolabeled mbr probe. The rearranged bands are indicated by arrows.

Lanes 1-3 were SstI digested Sample A, B and C respectively. Lanes 4-6 were HindIII digested Sample A, B and C respectively. Lanes 7-10 were DNA samples obtained from Patient 1 and were digested with enzyme HindIII (Lane 7: pretreatment bone marrow sample obtained in October of 1985; Lane 8: remission blood sample obtained in June of 1986; Lane 9: remission marrow sample obtained in June of 1986); Lane 10: remission marrow sample obtained in September of 1986). Lane 11 and 12 were DNA samples obtained from Patient 2 and were digested with HindIII. (Lane 11: pretreatment lymph node sample obtained in January of 1986; Lane 12: remission marrow sample obtained in October of 1986).

These rearranged bands in Sample A and C also comigrated with the immunoglobulin JH gene, which confirmed that breakpoint on chromosome 14 relocated in the J_H region. These three samples were subjected to PCR. The hybrid 18q21-J_H DNA sequences in Sample A were amplified and the signals were detected after the 15th cycle by radiolabeled primers 18q21 and J_H (Figure 3A). The amplified DNA segments also hybridized with

radiolabeled 18q21(+)_{II}, which further confirmed presence of the hybrid 18q21-J_H sequences (Figure 4, Lanes 1-3). Sample B was not amplifiable because there were no hybrid 18q21-J_H DNA sequences present. Sample C could not be
5 amplified because primer 18q21(+) was too far upstream (>1kb) from the breakpoint. Since approximately 60% of follicular lymphoma samples were mapped to have the t(14;18) breakpoint occurring within the mbr region (8,10), it was estimated that close to 60% of follicular
10 lymphomas will be amplifiable by the above described specific PCR technique.

Another manipulation was performed to show that the PCR-related method of the present invention could
15 preferentially amplify the hybrid 18q21-J_H DNA sequences, but not the normal DNA sequences. Dilutions of Sample A and Sample B in different ratios, 1:100, 1:5,000, and 1:100,000, were subjected to PCR. In the first two instances, the hybrid 18q21-J_H DNA sequences were
20 amplified and signals were detected after the 20th cycle of PCR as shown in Figure 3B and Figure 4, Lane 4. In the last instance (1:100,000 dilution), a strong and convincing signal was detected at the 30th cycle by using mixture of radiolabeled primers 18q21(+) and J_H (-) as a
25 probe (Figure 3C, Lane 3). The presence of the hybrid 18q21-J_H sequence was also further confirmed by the radiolabeled "internal" probe 18q21(+)_{II}.

Figure 3 shows a Southern blot analysis of PCR
30 amplified genomic DNA with radiolabeled primer 18q21(+) and primer J_H (-). Samples (1ug) of genomic DNA were dispensed in microcentrifuge tubes then denatured at 100°C for 5 minutes, centrifuged for 10 seconds to remove the condensation, and adjusted to 100 ul in a buffer
35 containing 10 mM tris, pH 7.5, 50 mM NaCl, 10 mM MgC₁₂, 1.5 mM deoxynucleotide triphosphate (each of 4 was used),

1 uM primer 18q21(+) and 1 uM primer JH(-). The samples were then transferred to a 42°C heat block for 2 minutes to allow annealing, followed by adding 1 ul of the Klenow fragment of E. coli DNA polymerase I (1 unit/ul) and 1 ul of 0.1 M dithiothreitol (DTT) and allowing extension of DNA sequences for 5 minutes. The cycle -- denaturation, reannealing and extension -- was repeated for 19 to 29 more times as needed, except that subsequent denaturations were performed at 95°C for 2 minutes. Amplified DNA (amplimers) from the PCR equivalent to 25 nanograms (ng) of the original DNA's was loaded on a 2% alkaline agarose minigel, fractionated by electrophoresis (50V) for 2 hours, and then neutralized and transferred to a nylon filter. Prehybridization, hybridization and washing of filters were carried out as described by Saiki *et al.* (14). A mixture of primer 18q21(+) and J_H(-) was radiolabeled with [γ -³²P]ATP to a specific radioactivity of >3uCi/pmol (microcurie/picomole) oligonucleotide and used as a probe. Autoradiography was carried out with a single intensification screen at -70°C for 48 hours. (A) 1 microgram (ug) of HindIII-digested Sample A (Lane 1) was compared with 25 ng of PCR amplified Sample A at various time points: the third cycle (Lane 2), the 7th cycle (Lane 3), the 10th cycle (Lane 4), the 15th cycle (Lane 5) and the 20th cycle (Lane 6). (B) Dilutions of Sample A and Sample B in different ratios (1:100 and 1:5,000) were subjected to PCR and 25 ng of PCR amplified DNA's were collected at various time points: Lanes 1-4 represent mixture of 1:100 dilution amplified for 10, 15, 20 and 24 cycles respectively; Lanes 5-8 represent mixture of 1:5,000 dilution amplified for 15, 20, 25, 30 cycles respectively. (C) Lane 1: Sample B subjected to PCR for 30 cycles; Lanes 2 and 3: Mixture of Samples A and B in 1:100,000 ratio amplified for 20 and 20 cycles of PCR respectively; Lanes 4-6: remission marrow and blood samples were obtained from Patient 1 and

subjected to PCR for 27 cycles, corresponding to samples shown in Figure 2, Lanes 8 - 10 respectively; Lane 7: pretreatment tumor sample obtained from Patient 2 and amplified for 20 cycles of PCR: Lane 8: remission marrow sample obtained from Patient 2 and subjected to PCR for 27 cycles. The ϕ X174 HaeIII molecular weight markers are labeled on the left of each autoradiograph.

Figure 4 shows a Southern blot analysis of PCR amplified genomic DNA with radiolabeled oligonucleotide 18q21(+)II. The PCR, radiolabeling, hybridization condition and washing condition, autoradiography were carried out as described above except that the radiolabeled oligonucleotide 18q21(+)II with specific activity of >3uCi/pmol was used and a probe and autoradiography was carried out for 20 hours. Lanes 1, 2 and 3: 25 ng of Sample A's were amplified for 20, 25 and 30 cycles respectively. Lane 4: 25 ng of mixture of Sample A and Sample B in 1:5,000 dilution were subjected to PCR for 40 cycles. Lane 5 and 6: 25 ng of remission marrow samples obtained from Patient 1 in June and September of 1986 respectively were amplified for 40 cycles. The ϕ X174 HaeIII molecular weight markers are labeled on the left of the autoradiograph shown in Figure 4.

Sequential follow-up studies in two patients with follicular lymphoma and the t(14;18) translocation were performed. The pretreatment tumor samples were shown to have chromosomal breakpoints occurring within mbr (Figure 2B, Lanes 7 and 11). The remission marrow and blood samples obtained from these two patients were first analyzed by morphological examination and conventional Southern blot hybridization with a radiolabeled mbr probe. All the samples appeared normal since none of them demonstrated any morphologic abnormality or rearranged

bands (Figure 2B, Lanes 8-10 and 12). However, hybrid DNA sequences were markedly amplified and thus clearly detected by the PCR technique in samples obtained from Patient 1 (Figure 3C, Lanes 4-6 and Figure 4, Lanes 5 and 6) indicating the presence of residual neoplastic cells carrying the t(14;18). Even though the hybrid 18q21-J_H DNA sequences were amplifiable in the pretreatment tumor sample obtained from Patient 2 (Figure 3C, Lane 7), no hybrid DNA sequences were detected in the remission marrow sample by the PCR technique (Figure 3C, Lane 8). These findings indicated that the concentration of neoplastic cells carrying the t(14;18) was too low to be detected by our current techniques or the patient was completely free of tumor and there was no t(14;18) target DNA sequences present for amplification.

In addition to the major breakpoint clustering region (mbr) observed in about 60% of follicular lymphoma, a new breakpoint clustering region (mcr) in the t(14;18) translocation has recently been described in about 30% of follicular lymphomas (16). Using proper primers derived from this region, the technique of the present invention may be applied to samples with the t(14;18) breakpoint occurring within this region. Furthermore, the t(14;18) has also been observed in about 20-40% of diffuse large cell lymphoma and 20% of small noncleaved cell lymphoma (9,16). Therefore, this technique can also be applied to subtypes of malignant lymphomas with the t(14;18) translocation besides follicular lymphoma. Finally, this technique can be applied to any chromosomal translocation if the breakpoints on both chromosomes are limited to a small DNA segment (about one kilobase), such as the t(11;14) translocation.

Utilizing the PCR technique of the present invention in a preferred application, a fundamental and very

important question was addressed, i.e., were there small numbers of circulating cells carrying the t(14;18) in patients with follicular lymphoma in early clinical stages of disease and in clinical remission?

5

Ten blood samples from patients with follicular lymphoma and the t(14;18) breakpoint within the mbr region were initially selected. These samples were obtained in various clinical states: 3, before treatment, 2, at partial remission and 5, at complete remission. Morphologically these samples appeared normal. They were analyzed both by the PCR assay and Southern blot analysis (Table I).

15

TABLE I

DETECTION OF LYMPHOMA IN PERIPHERAL
BLOOD BY THE PCR TECHNIQUE

20

CORRELATION WITH SOUTHERN BLOT
ANALYSIS ACCORDING TO CLINICAL STATUS

25	CLINICAL STATUS	PATIENTS STUDIED	POSITIVE BY PCR	POSITIVE BY SOUTHERN
	BEFORE TREATMENT	3	3	1
30	PARTIAL REMISSION	2	2	0
35	COMPLETE REMISSION	5	4	1
	TOTAL	10	9	2

40 Among the 10 samples analyzed, 9 were positive for the translocation by the PCR assay. In contrast, clonal bcl-2 gene rearrangement could be detected in only 2 cases by traditional Southern blot analysis. Three out of 3

samples obtained before treatment and 2 out of 2 samples obtained at partial remission were positive by the PCR assay. These findings indicated that when there was clinically detectable disease, there were small numbers of cells carrying the t(14;18) in circulation. The most intriguing finding was that even when patients achieved complete remission, circulating cells carrying the t(14;18) were detected in 4 out of 5 instances by the PCR assay.

10

The technique of the present invention was also used to determine whether there were circulating cells with the t(14;18) in an early clinical stage of the disease. As shown in Table II, three pretreatment blood samples from 3 patients whose lymph nodes were known to have the t(14;18) breakpoint within the mbr region were analyzed.

TABLE II

20

DETECTION OF LYMPHOMA IN PERIPHERAL
BLOOD BY THE PCR TECHNIQUE

CORRELATION WITH ANN ARBOR STAGE BEFORE THERAPY

25

ANN ARBOR STAGE	BREAKPOINT WITHIN mbr		BREAKPOINT NOT STUDIED	
	PATIENTS STUDIED	PATIENTS POSITIVE	PATIENTS STUDIED	PATIENTS POSITIVE
I	0	0	2	1
II	1	1	4	3
III	0	0	3	3
IV	2	2	1	1
TOTAL	3	3	10	8

40

All three of these samples were positive by the present PCR assay. In correlation with the clinical stage, one was from stage II disease and the other 2 were from stage IV disease. Ten pretreatment blood samples were analyzed from 10 patients for whom no DNA samples from the lymph nodes were available to study. Since about 60% of follicular lymphomas have the breakpoint within the mbr region, it was expected that approximately 60% of blood samples should be amplifiable if involving a lymphoma. As shown in Table II, 8 out of these 10 samples were positive. Even in the 6 samples obtained from clinical stage I or stage II disease, 4 were positive. The reason for the remaining being negative was most likely due to absence of a breakpoint within the mbr region. These findings strongly indicated that follicular lymphoma was a systemic disease despite the fact that the disease might be localized clinically.

To study the mechanism of frequent recurrences observed in follicular lymphoma, blood samples obtained from patients in clinical remission at various time points of the remission were analyzed by the PCR assay. As shown in Table III, five samples were obtained from patients at the time of remission of less than one year and who were known to have a breakpoint within the mbr region.

TABLE III

DETECTION OF LYMPHOMA IN PERIPHERAL BLOOD
BY THE PCR TECHNIQUE IN COMPLETE RESPONDERS

CORRELATION WITH DURATION OF COMPLETE REMISSION

<u>STUDIED</u> <u>YEARS IN</u> <u>CONTINUOUS</u> <u>REMISSION</u>	<u>BREAKPOINT WITHIN MBR</u>		<u>BREAKPOINT NOT</u>	
	<u>PATIENTS</u> <u>STUDIED</u>	<u>PATIENTS</u> <u>POSITIVE</u>	<u>PATIENTS</u> <u>STUDIED</u>	<u>PATIENTS</u> <u>POSITIVE</u>
>1 YEAR	5	4	1	1
2-3 YEARS	0	0	3	2
TOTAL	5	4	4	3

Four of these 5 patients showed evidence of subclinical disease by the PCR assay. Similarly in the 4 cases in whom the breakpoint was not studied, 3 were positive. In these 3 instances, 2 had been in remission for more than 2 years. These findings suggested that continuous relapse of follicular lymphoma may be due to recurrence of minimal residual disease which progresses slowly.

It may be that the PCR assay can reliably predict the likelihood of future relapse patients in remission. There were 5 complete responders in remission who had been followed clinically for more than 8 months after the results of the PCR assay were available. As shown in Table IV, in the 4 patients whose remission blood samples were positive, 3 have relapsed: 2 relapsed clinically and 1 developed clonal bcl-2 gene rearrangement as shown by Southern blot procedures.

TABLE IV

DETECTION OF LYMPHOMA IN PERIPHERAL BLOOD
BY THE PCR TECHNIQUE IN COMPLETE RESPONDERS

5

CLINICAL OUTCOME
ACCORDING TO RESULTS OF PCR ASSAY

10

RESULTS OF PCR ASSAY	NUMBER OF PATIENTS	OUTCOME	
		RELAPSE	REMISSION
15 POSITIVE	4	3	1
NEGATIVE	1	0	1

20 The remaining one patient is still in remission while on maintenance therapy. The only one patient whose remission blood sample was negative has been in continuous remission for more than 20 months. Since the pretreatment sample was amplifiable in this patient, possible

25 explanations for the remission sample being negative may be that either the number of neoplastic cells was too small to be detected or the patient was completely free of tumor.

30 The feasibility of using the PCR technique to detect minimal numbers of neoplastic cells carrying a chromosomal translocation was established by the methods of the present invention. Detection of small numbers of circulating monoclonal B cells by flow cytometry or clonal

35 immunoglobulin gene rearrangement in patients with follicular lymphoma in remission has been reported (1,17). The sensitivity of the methods of the present invention far exceeds the sensitivity limit achieved by conventional Southern blot analysis or the flow cytometric method.

40 Detection of minimal neoplastic cells with chromosomal translocation by means of PCR will make it possible to address several important biological and clinical

questions that could not be answered before. For example, do patients in long term remission have quiescent tumor cells with proliferative potential? Can detection of minimal residual tumor cells predict early relapse? Do
5 patients with persistent minimal residual disease after prolonged treatment require non-cross resistant therapy to prevent relapse? The answers will help in understanding tumor biology and designing strategies for cancer treatment.

The following cited articles are incorporated by reference herein for the reason cited.

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- 35 14. RK Saiki et al., Science, 230:1350 (1985).
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45

Changes may be made in the operation and arrangement of the various elements, steps and procedures described herein without departing from the concept and scope of the invention as defined in the following claims.

CLAIMS:

1. A method for detecting neoplastic cells in an individual, said neoplastic cells being characterized by a DNA translocation related to a specific breakpoint clustering region, the method comprising the steps of:

incubating a mixture comprising deoxyribonucleotide triphosphates, DNA from a cell sample of the individual, DNA polymerase, a first oligonucleotide primer and a second oligonucleotide primer, said first primer being identical to the upstream nucleotide sequence flanking the coding DNA translocation derived from one chromosome and said second primer being complementary to the downstream nucleotide sequence flanking the coding DNA translocation derived from the other chromosome, said incubating promoting annealing of the primers to a crossover site of the translocation and synthesis of coding or non-coding translocation DNA sequences and a substantial portion of flanking DNA sequences, the incubation then being terminated by DNA denaturation;

repeating said annealing, synthesis and denaturation in cyclic manner to facilitate duplication of the original and newly synthesized translocated and substantial flanking DNA sequences, said repeating being carried out in a manner allowing exponential amplification of DNA sequences between and including DNA regions complementary to the first and second oligonucleotide primers; and

35 .

probing the exponentially amplified DNA for the presence of oligonucleotide sequences characterizing the translocation region and identifying by positive probes the presence of neoplastic cells in the sample of the individual's cells.

2. A method for detecting neoplastic cells in an individual, said neoplastic cells being characterized by DNA translocation related to one or more specific breakpoint clustering regions, the method comprising the steps of:
- incubating a mixture comprising deoxynucleotide triphosphates, original DNA from cells of the individual, DNA polymerase and at least one set of oligonucleotide primers, each set of oligonucleotide primers consisting of a first oligonucleotide primer and a second oligonucleotide primer, said first oligonucleotide primer being identical to an upstream coding nucleotide sequence flanking a DNA breakpoint clustering region and said second primer being complementary to a downstream coding nucleotide sequence flanking this DNA breakpoint clustering region, said non-coding DNA base-pairing with the coding DNA, said incubating being under conditions promoting annealing of the primers to the crossover sites of the translocation and synthesis of coding and non-coding translocation DNA and including a substantial portion of flanking DNA, the incubation then being terminated by DNA denaturation;

- repeating said annealing, synthesis and denaturation
in a cyclic manner to facilitate further
synthesis of the original DNA and of newly
synthesized DNA, said repeating being carried
out in a manner allowing repeated primer
extensions and exponential amplification of DNA
sequences at the crossover sites of the
translocations; and
- probing exponentially amplified DNA for the presence
of oligonucleotide sequences characterizing the
translocations and identifying by positive
probes the presence of neoplastic cells in the
sample of the individual's cells.
3. The method of claim 1 wherein the first and second
primer nucleotide sequences are located within about 1000
bases from the nearest breakpoint clustering region.
4. The method of claim 1 wherein the oligonucleotide
primers are each defined further as being from between
about 12 and about 20 nucleotides in length.
5. The method of claim 1 wherein there is a single
breakpoint clustering region.
6. The method of claim 1 or 2 wherein the translocation
is defined further as involving a t(14;18) translocation.
7. The method of claim 2 wherein there are two or more
breakpoint clustering regions to be analyzed.

8. The method of claim 2 wherein there is a major breakpoint clustering region and a minor breakpoint clustering region.

5

9. The method of claim 8 wherein said major breakpoint clustering region is the mbr region and said minor breakpoint clustering region being the mcr region.

10

10. The method of claim 2 wherein there are at least two breakpoint regions to be analyzed, two sets of oligonucleotide primers are added to the mixture and the probing is defined further as involving probes with
15 sequences derived from the crossover sites of the translocated DNAs.

11. The method of claim 1 or 2 wherein the probing step
20 is defined further as involving Southern blot analysis.

12. The method of claim 1 or 2 wherein the incubating step is followed by a terminating step and these steps are
25 repeated from about 20 to about 50 times to exponentially amplify DNA flanked by oligonucleotide primers.

13. The method of claim 1 or claim 2 wherein the
30 extension of a primer includes synthesis of translocation sequences and a substantial flanking DNA region including that nucleotide sequence complementary to the other primer.

35

14. The method of claim 12 wherein the terminating step involves denaturation of DNA templates.
- 5 15. The method of claim 1 or 2 wherein the neoplasm is human follicular lymphoma.
- 10 16. The method of claim 1 or 2 wherein the neoplasm is a non-Hodgkin's lymphoma.
- 15 17. The method of claim 1 or 2 wherein the neoplasm is a B-cell lymphoma.
18. The method of claim 1 or 2 wherein the neoplasm is a large cell lymphoma, a diffuse large cell lymphoma, a small noncleaved cell lymphoma, a B-cell leukemia or a
20 Pre-B-cell leukemia.
- 25 19. The method of claim 1 or 2 wherein the cell sample of the individual comprises neoplastic cells.
20. The method of claim 1 or 2 wherein the deoxynucleotide phosphates include deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytosine
30 triphosphate and deoxyguanosine triphosphate.
21. The method of claim 1 or 2 wherein the DNA polymerase is the Klenow fragment of E. coli DNA polymerase or
35 Thermus aquaticus DNA polymerase.

22. The method of claim 1 or 2 wherein the primers are at a concentration in excess of that needed to bind complementary oligonucleotide sequences of the DNA.

5

23. The method of claim 1 or 2 wherein the primers are at about 1 micromolar concentration.

10 24. The method of claim 1 or 2 wherein the chromosomal translocation is characterized by the breakpoints at both chromosome clustering within several hundred base pairs.

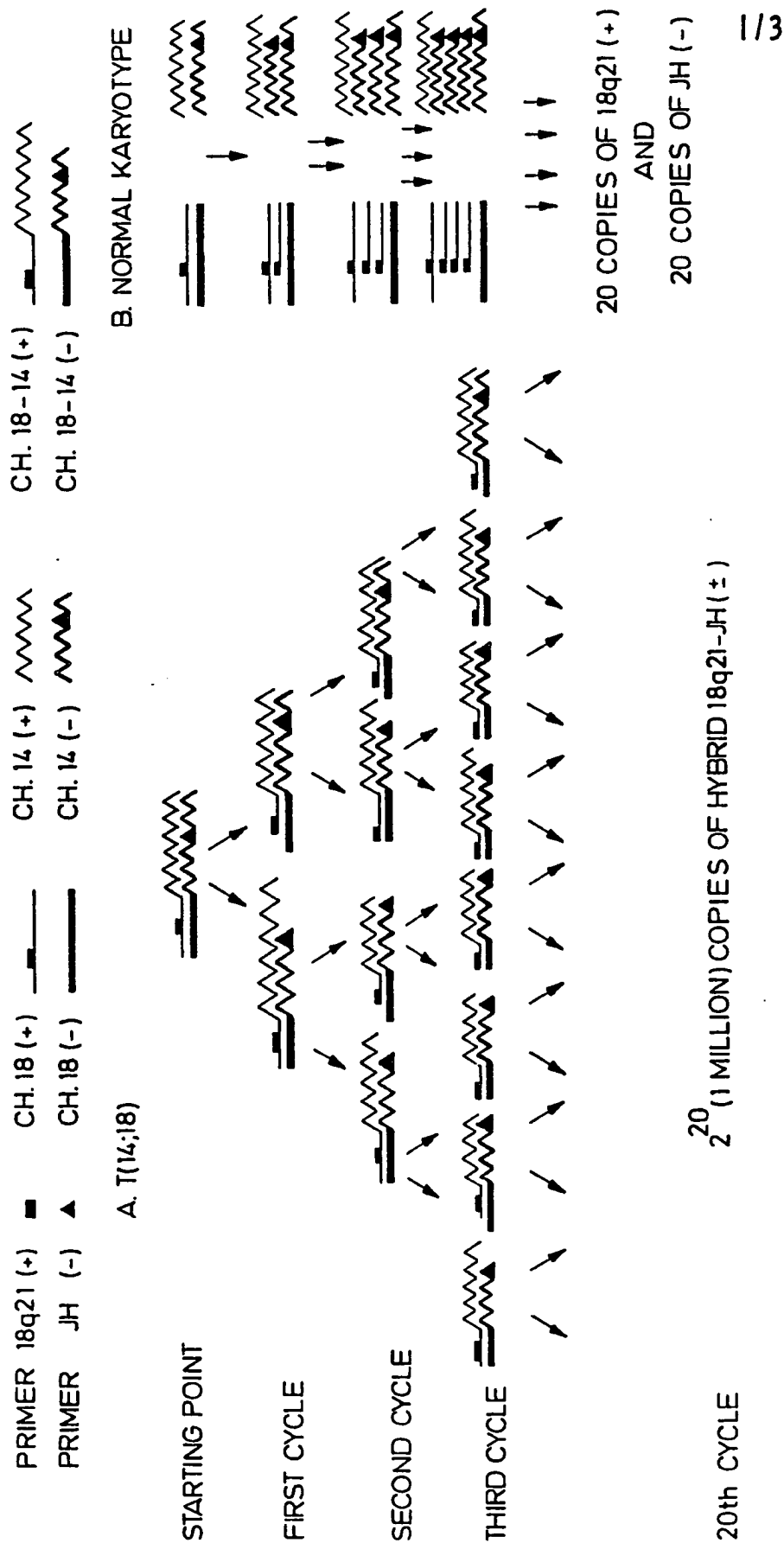


Fig. 1

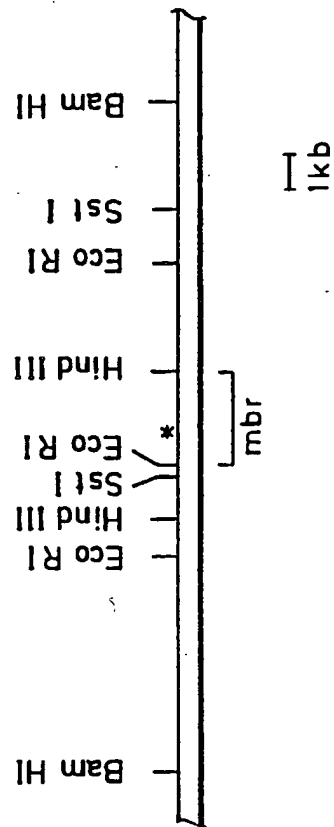


Fig. 2A

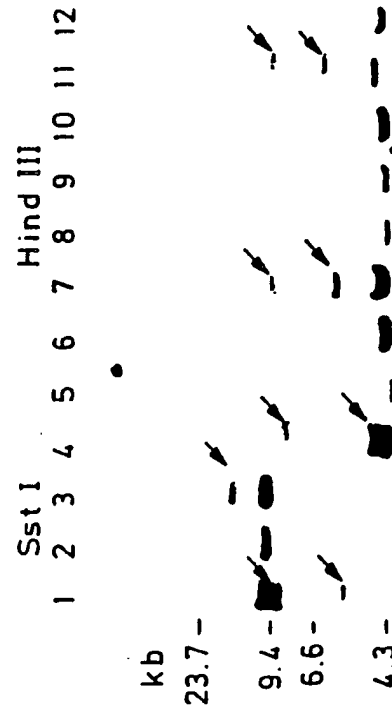


Fig. 2B

3/3

Fig. 3A

1 2 3 4 5 6



Fig. 3B

1 2 3 4 5 6 7 8



Fig. 3C

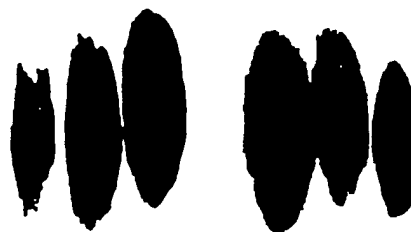
1 2 3 4 5 6 7 8



Fig. 4

bp 1 2 3 4 5 6

1,353 -
1,078 -
872 -
603 -
310 -



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00843

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 Q 1/68, //C 07 H 21/04														
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; vertical-align: top;">IPC⁴</td> <td style="border: none; vertical-align: top;">C 12 Q</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	C 12 Q								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category ⁹</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">A</td> <td style="vertical-align: top;">Science, vol. 230, 20 December 1985, R.K. Saiki et al.: "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", pages 1350-1354, see the whole article --</td> <td style="vertical-align: top;">1,2,20-24</td> </tr> <tr> <td style="vertical-align: top;">P,X</td> <td style="vertical-align: top;">Proc. Natl. Acad. Sci. USA, vol. 85, July 1988, M. Crescenzi et al.: "Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease", pages 4869-4873, see the whole article --</td> <td style="vertical-align: top;">1-24</td> </tr> <tr> <td style="vertical-align: top;">P,X</td> <td style="vertical-align: top;">Chemical Abstracts, vol. 110, no. 3, 16 January 1989 (Columbus, Ohio, US), S.-S. Maryalice et al.: "Detection of occult follicular lymphoma by specific DNA amplification", page 147, abstract no. 19393s, & Blood 1988, 72(5), 1822-5, see abstract --</td> <td style="vertical-align: top;">1-24</td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	Science, vol. 230, 20 December 1985, R.K. Saiki et al.: "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", pages 1350-1354, see the whole article --	1,2,20-24	P,X	Proc. Natl. Acad. Sci. USA, vol. 85, July 1988, M. Crescenzi et al.: "Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease", pages 4869-4873, see the whole article --	1-24	P,X	Chemical Abstracts, vol. 110, no. 3, 16 January 1989 (Columbus, Ohio, US), S.-S. Maryalice et al.: "Detection of occult follicular lymphoma by specific DNA amplification", page 147, abstract no. 19393s, & Blood 1988, 72(5), 1822-5, see abstract --	1-24
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<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search <div style="text-align: center;">21st June 1989</div> </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center;">12 JUL 1989</div> </td> </tr> <tr> <td style="width: 50%; border: none; vertical-align: top;"> International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; border: none; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: right;">P.C.G. VAN DER PUTTEN</div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center;">21st June 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center;">12 JUL 1989</div>	International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: right;">P.C.G. VAN DER PUTTEN</div>								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Science, vol. 237, 10 July 1987, M.-S. Lee et al.: "Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification", pages 175-178, see the whole article --	1-24
A	Science, vol. 228, 21 June 1985, Y. Tsujimoto et al.: "Involvement of the bcl-2 gene in human follicular lymphoma", pages 1440-1443, see the whole article --	1-19
A	Proc. Natl. Acad. Sci. USA, vol. 82, November 1985, M.L. Cleary et al.: "Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18", pages 7439- 7443, see the whole article --	1-19
A	Chemical Abstracts, vol. 107, no. 5, 3 August 1987 (Columbus, Ohio, US), M. Raffeld et al.: "Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region", page 186, abstract no. 34198p, & Cancer Res. 1987, 47(10), 2537-42, see the whole abstract --	1-19
A	EP, A, 0236069 (CETUS CORP.) 9 September 1987, see pages 1,2; pages 24-53 ----	1,2,20-24

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